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Investigating the eNOS and IFN-γ Gene Variants Susceptible to Bipolar Disorder or Schizophrenia in a Turkish Cohort

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Abstract

Background: Schizophrenia (Sch) and bipolar disorder (BD) are debilitating chronic psychiatric disorders that are both etiologically and clinically heterogeneous. According to the gathered evidence, multiple mental disorders are accompanied by inflammation. Interferon-γ (IFN-γ), as a regulatory cytokine, is involved in the immune response as a proinflammatory mediator. Several critical physiological functions are regulated and governed by nitric oxide (NO) in the central nervous system. This study aimed to investigate the association between IFN-γ +874T/A and eNOS 894G/T variants and Sch or BD susceptibility.

Methods: Blood samples were collected from patients and healthy subjects. IFN-γ +874T/A and eNOS 894G/T variants were genotyped with the PCR-RFLP. We evaluated the patients with some clinical parameters (the duration of the disorder, age of onset, number of hospitalizations, family history, tobacco smoking or drug, alcohol usage). Statistical analyses were performed using the SPSS version.

Results: When the genotype distributions and allele frequencies of the IFN-γ +874T/A and eNOS 894G/T in the patients diagnosed with Sch or BD were compared with the control group, there were not found to be significant differences between the groups. When comparing IFN-γ +874T/A and eNOS 894G/T genotype distributions and allele frequencies of Sch or BD patients due to clinical parameters, the genotype distribution of IFN-γ +874T/A in BD patients was significantly different between the groups due to the presence of tobacco smoking (OR: 0.217, 95%Cl: 0.054–0.878; p = 0.032).

Conclusions: To the best of our knowledge, this is the first study that examines the association between the IFN-γ and eNOS gene variants and Sch or BD in a Turkish population. Although IFN-γ +874T/A and eNOS 894G/T variants are not considered as candidate genes for Sch or BD, the results indicated that the BD patients carrying IFN-γ +874T/A AA genotype were less susceptible to tobacco smoking in a Turkish population.

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INTRODUCTION

One of the most common damaging psychiatric disorders, which have a morbidity risk of 0.5%–2.7% during life, is Schizophrenia (Sch) [1]. Its characteristics are three broad-spectrum behavioral domains, including positive symptoms such as hallucinations and delusions and negative symptoms such as anhedonia, social withdrawal, cognitive domain, and apathy [2]. The etiopathogenesis of Sch is explained by the hypotheses related to neuroimmunological, neurodevelopmental, and genetic neurotransmitters [3]. Bipolar disorder (BD) is a severe, chronic, and disabling disease. It is estimated that its prevalence during life is 2.4% [4]. It is cardinally diagnosed by at least one hypomania or mania episode despite the depressive episodes' predomination during the illness.

Increased proinflammatory cytokines obtained from the growing body of evidence show a relationship between the immune-mediated mechanisms and the neurobiology of psychiatric disorders [5]. A regulatory cytokine, i.e., Interferon-γ (IFN-γ), which is involved in the immune response, acts as a proinflammatory mediator. Human *IFN-γ*, which has four exons with an approximate span of 6 kb, is located on chromosome 12 (12q14). In vitro transcription of *IFN-γ* increases through a change of T to A in the +874 (rs2430561) position from the site of the translation start in the first intron of the *IFN-γ* gene [6]. Nitric Oxide Synthases (NOSs) catalyzes the oxidation of L-arginine to Nitric Oxide

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(NO). Endothelial NOS (eNOS), an isoform of enzymes producing NO, is expressed constitutively in endothelial cells [7]. NO, which participates in the pathophysiology of several psychiatric disorders such as BD and Sch, is an important neurotransmitter. The presence of a relationship between NOS activity and Sch is also supported by the researchers finding a higher NO in plasma of schizophrenics and post-mortem brain tissue [8]. NO levels in the blood have been used in several studies as biomarkers to detect BD [9]. There is a correspondence between a Glu-Asp change at codon 298 and a functional polymorphism in exon 7 of the human *eNOS* gene. *eNOS G894T* T allele carriers exhibit the diminished activity of the eNOS enzyme compared to GG homozygotes [10]. Studies examining the relationship between promoter polymorphism in IFN-γ and eNOS – encoding genes and Sch or BD development have been scarce. Considering the potential role of IFN-γ and eNOS in psychiatric disorders such as Sch or BD, the linkage studies pointing to the chromosomal region containing the IFN-γ gene and eNOS gene the proposed inflammatory imbalance, the present research focused on examining the association between IFN-γ gene polymorphism at position +874T/A and eNOS gene polymorphism at position 894G/T the occurrence of Sch or BD among the Turkish population. Therefore, we hypothesized that these gene variants could be associated with the pathogenesis of Sch or BD. To our knowledge, this is the first clinical research comparing distributions of IFN-γ +874T/A and eNOS 894G/T variants in Turkish patients with Sch or BD according to clinical parameters (the duration of the disorder, age of onset, number of hospitalizations, family history, and tobacco smoking or drug, alcohol usage) in detail.

This study aimed to investigate the association between *IFN-γ +874T/A* and *eNOS 894G/T* variants and Sch or BD susceptibility by comparing these individuals to healthy controls considering clinical parameters.

METHODS

Study Population

This research was designated as a cross-sectional study. Subjects in the association study were 118 patients with Sch, 104 patients with BD, and 100 healthy controls. The patients consecutively were admitted to the Department of Psychiatry in Bakirkoy Research and Training Hospital for Psychiatry, Neurology, and Neurosurgery for six months. The diagnosis was assigned independently by two experienced senior psychiatrists based on the Diagnostic and Statistical Manual of Mental Disorders-IV [11]. The patients who followed up from the community mental health center were receiving regular treatment and remission. Healthy controls were recruited from the same geographical areas as the patients, and they were well-matched with the patients' group in terms of gender, age, and ethnicity. The control group who did not have any psychological disease was selected. A sociodemographic and clinical characteristics data form is a detailed interview that includes questions about clinical information such as family history, comorbid disease history, and complaints related to Sch or BD and was prepared by the researchers. The study was approved by the Local Ethics Committee of Hospital (07.11.2017/81). All participants were given detailed verbal and written information regarding the purpose and procedures of the study, and their written consent was obtained. This study was conducted under guidelines laid down in the Declaration of Helsinki, and the Local Ethics Committee approved all procedures involving human subjects.

Inclusion and Exclusion Criteria

According to the SCID-I interview, subjects of 18 to 65 years of age, of either gender, were literate, agreed on the participation, and were diagnosed with Sch or BD. They had no other systemic/neurological disease that may affect cognitive functions (dementia, epilepsy, Parkinson disease, head trauma accompanied by loss of consciousness) included in the study. We had excluded subjects who had mental retardation, neurodevelopmental disorders such as autism, a diagnosis of axis-1 disorder other than Sch and BD as a result of the SCID-I interview, Sch, or BD secondary to a general medical condition, dementia, or brain damage.

Genotyping Analysis

About 5 mL peripheral blood was collected through venipuncture using Vacutainer tubes with EDTA as an anticoagulant to analyze these variants. DNA was extracted from leukocytes according to the established protocol [12]. The extracted DNA was stored at – 20°C until the analysis was completed. Polymorphism of the *IFN-γ +874T/A* was determined by the allele-specific polymerase chain reaction (PCR) method. For each allele, PCR reaction was carried out on a DNA template with a pair of specific primers (reverse: TCA ACA AAG CTG ATA CTC CA; forward +874T: TTC TTA CAA CAC AAA ATC AAA TCT or forward +874A: TTC TTA CAA CAC AAA ATC AAA TCA; amplimer length, 262 bp), 25 µl total volüme reaction mix (provided by the manufacturer), and Tth polymerase (Epicentre Biotechnologies, Madison, WI, USA). To analyze the *eNOS 894G/T* polymorphism, PCR was used to amplify a 206-bp fragment (forward primer 5'-GGCTGGACCCCAGGAAA – 3'; reverse primer 5'-CACCCAGTCAATCCCTTTGGT-3'). The resulting fragment was digested with MboI restriction endonuclease (Invitrogen CA, USA) overnight at 37°C. Digestion was resolved on a 3% agarose gel and visualized under ultraviolet light [13, 14].

Statistical Analysis

All data were analyzed using software SPSS version 22.0 for Windows (SPSS Inc., Chicago, IL; USA). Quantitative data (clinical parameters and *IFN-γ +874 T/A* or *eNOS 894G/T* genotype) represented as descriptive statistics included mean, standard deviation, and percentages. The comparison of *IFN-γ +874 T/A* and *eNOS 894G/T* genotype and allele distributions of Sch or BD patients with the control group were analyzed by the Pearson chi-square

test or Fisher's exact test. Age-and sex-adjusted odds ratios and 95% confidence intervals calculated. The power analysis was performed with the "G*power" software (version 3.0.5, http://www.psycho.uni-duesseldorf.de/abteilungen/aap/gpower3/), post hoc goodness of fit x2 test, with an "-error" probability of 0.05. All analyses were two-tailed, and differences were interpreted as statistically significant when $p < 0.05$.

RESULTS

IFN-γ +874T/A genotyping

In the present study, a total of 322 subjects, including 118 Sch patients, 104 BD patients, and 100 healthy adult controls, were evaluated according to their sociodemographic and clinical characteristics, as shown in Table 1. According to the *IFN-γ +874T/A* genotype distribution, 22 (19%) of the patients diagnosed with Sch had TT, 58 (59%) had AT, and 36 (32%) had AA genotypes. 20 (19.2%) of the patients diagnosed with BD had TT, 49 (47.1%) had TA, and 35 (33.7%) had AA genotypes. When we compared the genotype distribution and allele frequency of the IFN-γ +874T/A in the patients diagnosed with Sch or BD with the control group, we did not found significant differences between the groups (p>0,05) (Table 2).

Table 1. The clinical parameters and genotype distributions of Sch or BD patients.

		Sch (N:118)	BD (N:104)
		n (%)	n (%)
Sex	Female	31 (26.3)	62 (59.6)
	Male	87 (73.7)	42 (40.4)
Family History	No	69 (58.5)	51 (49)
	Yes	49 (41.5)	53 (51)
	TT	22 (19)	20 (19.2)
IFN-γ+874 T/A	AT	58 (59)	49 (47.1)
	AA	36 (32)	35 (33.7)
	GG	68 (57.6)	55 (52.9)
eNOS 894G/T	GT	44 (37.3)	45 (43.3)
	TT	6 (5.1)	4 (3.8)
		Mean ± SD	Mean±SD
Age		40.7±10.3	41.4±11.5
Age of onset (year)		24,44±8,17	25.6±8.5
The number of hospitalization		3,55±4,67	3.28±4.2

Sch: schizophrenia; BD: bipolar disorder; SD: standard deviation.

Table 2. Comparison of IFN-γ+874 T/A genotype and allele distributions of Sch or BD patients with the control group.

IFN-γ+874 T/A	Sch	Controls	OR Exp (B)	95% CI	p
Genotypes	n= 116 (%)	n=100 (%)			
TT	22 (19)	21 (21)	0.880[a]	0.451-1.718[a]	0.735[a]
AT	58 (59)	55 (55)	1.033*	0.508-2.102*	0.928*
AA	36 (32)	24 (24)	1.619*	0.723-3.627*	0.242*
Alleles					
T	102 (44)	97 (48.5)	1.200[a]	0.821-1.754[a]	0.384[a]
A	130 (56)	103 (51.5)			
IFN-γ +874T/A	BD	Controls	OR Exp(B)	95% CI	p
Genotypes	n= 104 (%)	n=100 (%)			
TT	20 (19.2)	21 (21)	0.896[a]	0.451-1.777[a]	0.862[a]
TA	49 (47.1)	55 (55)	0.927*	0.447-1.922*	0.838*
AA	35 (33.7)	24 (24)	1.591*	0.704-3.593*	0.264*
Alleles					
T	89 (42.8)	97 (48.5)			
A	119 (57.2)	103 (51.5)	1.234[a]	0.835-1.824[a]	0.320[a]

Sch:schizophrenia; BD: bipolar disorder; OR: odds ratio; CI: confidence interval; *Pearson chi-square; [a]Fisher's Exact Test; OR (95%CI) was adjusted by age and sex.

eNOS 894G/T Genotyping

According to the *eNOS 894G/T* genotype distribution, 68 (57.6%) of the patients diagnosed with Sch had GG, 44 (37.3%) had GT, and 6 (5.1%) TT genotypes. 55 (52.9%) of the patients diagnosed with BD had GG, 45 (43.3%) had GT, and 4 (3.8%) TT genotypes. When we compared the genotype distribution and allele frequency of the eNOS 894G/T in patients diagnosed with Sch or BD with the control group, we did not found significant differences between Sch or BD patients and healthy controls for eNOS 894G/T variant (p>0.05) (Table 3).

Table 3. Comparison of eNOS 894G/T genotype and allele distributions of Sch or BD patients with the control group.

eNOS 894G/T	Sch	Controls	OR Exp (B)	95% CI	p
Genotypes	**n= 118 (%)**	**n=100 (%)**			
GG	68 (57.6)	62 (62)	0.182*	0.021-1.561*	0.120*
GT	44 (37.3)	37 (37)	0.188*	0.021-1.643*	0.131*
TT	6 (5.1)	1 (1)	5.304[a]	0.628-44.817[a]	0.128[a]
Alleles					
G	180 (76.3)	161 (80.5)	0.779[a]	0.491-1.234[a]	0.297[a]
T	56 (23.7)	39 (19.5)			
eNOS 894G/T	**BD**	**Controls**	**OR Exp(B)**	**95% CI**	**p**
Genotypes	**n= 104 (%)**	**n=100 (%)**			
GG	55 (52.9)	62 (62)	0.179*	0.020-1.599*	0.124*
GT	45 (43.3)	37 (37)	0.244*	0.027-2.202*	0.209*
TT	4 (3.8)	1 (1)	3.960[a]	0.435-36.057[a]	0.369[a]
Alleles					
G	155 (74.5)	161 (80.5)	0.674[a]	0.945-1.074[a]	0.101[a]
T	53 (25.5)	39 (19.5)			

Sch:schizophrenia; BD: bipolar disorder; OR: odds ratio; CI: confidence interval; *Pearson chi-square; [a]Fisher's Exact Test; OR (95%CI) was adjusted by age and sex.

Comparison of Genotype Distributions of Ifn-Γ +874t/A and Enos 894g/T Genotype Of Sch or Bd Patients Due to Clinical Parameters:

Comparing of IFN-γ +874T/A genotype distribution of Sch or BD patients due to clinical parameters (the duration of the disorder, age of onset, number of hospitalizations, family history, and tobacco smoking or drug, alcohol usage), the genotype distribution of BD patients was significantly different between the groups due to the presence of tobacco smoking. AA genotype was found significantly higher in the non-smoking BD group than the smoking BD group (OR: 0.217, 95%Cl: 0.054–0.878; p = 0.032). When comparing *eNOS 894G/T* genotype distribution of Sch or BD patients due to clinical parameters, there was not found to be a significant difference between the groups (p>0,05) (data not shown) (Table 4).

Table 4. Comparison of IFN-γ+874 T/A genotype and allele distributions of BD patient groups based on tobacco smoking status

IFN-γ +874T/A	BD Patients (Non-smoker)	BD Patients (Smoker)	OR Exp (B)	95% CI	p
Genotypes	n=54 (%)	n=50 (%)			
TT	10 (18.6)	9 (18)	0.966[a]	0.357-2.615[a]	1.000[a]
TA	20 (37.0)	29 (58)	1.262*	0.394-4.037*	0.695*
AA	24 (44.4)	12 (24)	0.217*	0.054-0.878	**0.032***
Allele					
T	40 (37)	47 (47)	0.663[a]	0.381-1.154[a]	0.161[a]
A	68 (63)	53 (53)			

Sch:schizophrenia; BD: bipolar disorder; OR: odds ratio; CI: confidence interval; *Pearson chi-square; [a]Fisher's Exact Test; OR (95%CI) was adjusted by age and sex.

DISCUSSION

Sch and BD are debilitating chronic psychiatric diseases that are etiologically and clinically heterogeneous. The pathogenesis of Sch [15] and BD [16] includes infections and inflammation. According to the various case-control or meta-analyses researches, the Sch and BD patients show signs of a peripheral inflammation with low grade, upregulating many cytokines [15, 17, 18]. IFN-γ, a member of the type 2 class of interferons, is produced by CD4+ Th1, natural killer (NK), and CD8+ cytotoxic T cells and is a central mediator of the Type 1/Type 2 immune balance. IFN-γ has different roles, such as activation of the class II molecules of the major histocompatibility complex (MHC), an increase in the antigen macrophage and presentation and activity of NK cell, promotion of the leukocyte migration, and stimulation of the production of IgG3 and IgG2 [19]. The expression of IFN-γ was found significantly reduced in patients with Sch as compared to the normal controls [20]. Also, Arolt et al. reported that the production of IFN-γ decreased in Sch patients compared to the control group during treatment [21]. There is an association between several inflammatory and autoimmune diseases and a single nucleotide polymorphism (SNP) in the first intron of the human *IFN-γ* gene with nuclear factor-KB (NFKB)-binding region [22]. There is a T allele seen in high plasma IFN-γ, while there is an A allele in low plasma IFN-γ. In the present study, there was no statistically significant difference found between *IFN-γ +874T/A* polymorphism of the Sch patients with the control group. Therefore, we speculate that our results suggest that *IFN-γ +874T/A* polymorphism is not associated with the pathophysiology of Sch in the Turkish population. When the researches in the literature about IFN-γ +874T/A polymorphisms related to the Sch patients are reviewed, a study found the correlation between the allele A at position +874 in the *IFN-γ* gene and the risk of paranoid Sch development, which increases 1.66 fold in males, still, it does not like that in the Polish females [23]. Jemli et al., in a study on the Tunisian population, observed that *IFN-γ +874T/A* variant TT genotype and T allele showed higher frequencies in all paranoid Sch patients than those in the male controls [24]. However, another study found no significant association between genotype distribution of *IFN-γ +874T/A* variant and risk of Sch [25].

Again, when we reviewed the studies on the relationship between *IFN-γ* and BD, It was seen that IFN-γ/IL-4 ratios were significantly higher in the BD patients than those in the normal controls [26]. Also, the concentration of IFN-γ was higher in the BD patients during remission after depression than the healthy controls [27]. Nayeri et al. reported that *IFN-γ +874T/A* codominant model (T/T vs. T/A-A/A) and the dominant model (T/T vs. T/A-A/A) were associated with decreased BD risk in the Iranian population [22]. Yoon et al. found that the *IFN-γ +874T/A* variant T allele was significantly more common among patients with BD than in controls [28]. In our study, the genotype and allele frequencies of *IFN+874T/A* variant did not show any statistically significant difference between the Sch or BD patients and controls. However, since all Sch patients were tobacco smoking, we also evaluated BD patients as smoker and non-smoker. *IFN+874T/A* AA genotype was higher in the non-smoking BD patients than in the smoking BD patients. There is a relationship between the IFN-γ gene variant at position +874 and low (AA), intermediate (TA), or high (TT) IFN-γ secretion [29]. So, the BD patients carrying the AA genotype (associated with low IFN-γ) appear to be less predisposed to smoking. In the literature, César-Neto et al. reported that smoking increased both protein and mRNA levels of IFN-γ in gingival tissue [30]. Again, there was an association between the *IFN-γ* genotype and baseline of lung function, and this association was modified by tobacco smoking [31]. When Gangwar et al. investigated the association of IFN-γ +874 gene polymorphism with the risk of cervical cancer, they showed that the IFN-γ +874 AA genotype's frequency was higher in cervical cancer patients among tobacco users [32].

The *eNOS* encoding gene is located on chromosome 7q35-36 and includes 26 exons and 25 introns encoding a 135 KDa protein, with 1203 amino acids [33]. The *eNOS* gene is highly polymorphic. The most described variant, which is the 894G/ T variant located in exon 7, which was found to cause a reduction of NO synthesis [34]. The protein's primary structure may be altered by this variant, which can directly change the enzyme's functional properties. According to two different studies, the eNOS protein, which contains Asp at position 298, is exposed to selective proteolytic cleavage in vascular tissues and endothelial cells [35]. Some physiological functions are also mediated by NO, a gaseous messenger molecule in the nervous system. These functions include the release of mediators, development of nervous tissue, and regulation of synaptic plasticity [36]. According to Reif et al., there was a significant reduction in proliferation of neuronal progenitor cells in the dentate gyrus among the eNOS-deficient mice, suggesting that eNOS was influential in the stimulation of neuroneogenesis [37]. According to Chen et al., the expression of brain-derived neurotrophic factor is regulated by eNOS in the ischemic brain, affecting progenitor cell proliferation, neurite outgrowth, neuronal migration, and influencing the functional recovery after stroke [38]. In the present study, there was no significant association between the Sch or BD patients and the controls in terms of the *eNOS 894G/T* variant. In contrast to our study, Burghardt et al. found an association between the *eNOS T−786C* variant and endothelial functioning among the Sch patients taking atypical antipsychotics[7]. Again, in the Iranian population, the *eNOS 894G/T* variant was associated with catatonic Sch patients [39]. Earlier investigations on BD found an increase in NO levels selectively in depressive episodes [40] and during different mood states [41]. Ikenouchi-Sugita et al. found the association between the plasma metabolites of NO and three polymorphisms of the eNOS among the patients suffering from a major depressive disorder and the healthy controls; even they did not observe an association between the polymorphisms of the eNOS gene and the

pathogenesis of depression as in our study [42]. Also, there was a significant association between eNOS 894G/T and BD in the Iranian population [43]. Reif et al. reported that all three polymorphisms of the *eNOS* gene were in significant linkage disequilibrium with each other and thus suggested that the *eNOS* genotype might convey a modest genetic risk of BD development [44].

The present study has several limitations. The first limitation of the present study is that the sample size of the groups is relatively small. Secondly, in our research, we included only Turkish subjects as the studied population. Further studies should be done on other ethnic communities because there is interethnic variability, and more studies are needed to confirm our findings.

In conclusion, although *IFN-γ +874 T/A* and *eNOS 894G/T* variants are not considered as candidate genes for Sch and BD, the results indicated that the BD patients carrying *IFN-γ +874 T/A* AA genotype were less susceptible to tobacco smoking in a Turkish population. There is still a need for further studies on the relationship between these variants and Sch and BD, mainly by studying the different clinical features.

Informed consent: We obtained written, informed consent from subjects and patients who participated in this study.

Statement of interest: All authors declare not to have any conflicts of interest that might be interpreted as influencing the manuscript's content.

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Contributions of Authors: SP, HMA, and AFN are responsible for the formulation of overarching research goals and aims. SP, HMA, HSC, and AFN conceived and designed the study. SP, HSC, and YO are responsible for the provision of study materials and laboratory samples. HMA and AFN drafted the manuscript. SP and MP supervised the study.

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